

Effect of Disinfectants on Variola Virus in Cell Culture

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Twenty kinds of disinfectants were examined for ability to inactivate variola virus. Cytopathic effect and plaque formation on monolayer cultures of an established monkey kidney cell line were used as indicators of virus inactivation. A micromethod using microplate cultures, and not requiring a CO₂ incubator, was adopted. The procedures were straightforward, showing good reproducibility. Among the compounds tested, several were found to be superior because of the minimum concentrations required for complete inactivation of virus. The purified viruses were shown to be more sensitive to the compounds than were the crude samples. The virus inactivation kinetics curves were determined by plaque counting. The usefulness of this method for quantitative analysis of disinfecting effect is suggested.

In view of the continuing danger of smallpox in various parts of the world, the necessity for searching out efficient disinfectants against variola virus is obvious. Whereas there are reports regarding the effect of disinfectants upon poxviruses (1, 2, 6-9, 14), problems remain to be further investigated. In this study we examined 20 kinds of compounds for their disinfecting effects upon variola virus, adopting a microplate cell culture method. Viral cytopathic effect (CPE) and plaque formation (11, 12) were indicators of virus inactivation.

MATERIALS AND METHODS

Viruses and cell cultures. Two strains of variola virus were used for testing: the Harvey strain, isolated by Downie and Dumbell (3), and the Bandung strain, isolated by us (10). Vaccinia viruses, Ikeda and Lister strains, were used for purposes of comparison. The viruses were propagated in monolayer cultures of an established cynomolgus monkey kidney cell line, Jinet (13). The culture medium for cellular growth consisted of Eagle minimal essential medium supplemented with 10% heat-inactivated bovine serum and 60 µg of kanamycin per ml; for maintenance of good cell growth, the bovine serum was replaced by 2% inactivated calf serum. The fluid from virus-infected cultures was centrifuged at 1,500 rpm for 20 min, and the supernatant was used as viral inoculum. In addition to the crude samples, the viruses were purified by repeated centrifugations at 12,000 rpm for 60 min, followed by resuspension in phosphate-buffered saline (4).

Microplate cultures were prepared as follows. The cells were trypsinized and suspended in the culture

medium. One-tenth-milliliter samples of the cell suspension were introduced into flat-bottomed wells of a plastic plate (Falcon Microtest II, 124 by 82 by 14 mm), which was then tightly sealed with a plate sealer and placed in an ordinary incubator at 35°C. The cellular sheets were formed 1 to 2 days thereafter. Just prior to use, the cultures were washed with minimal essential medium.

Disinfectants and assay of disinfecting activity. The disinfectants tested are listed in Table 1. Nine parts of each of the compounds, appropriately diluted in sterile distilled water, were mixed with one part of the virus suspension of known titer and left at room temperature (18 to 23°C). At a fixed time, portions of the mixture were taken and diluted 100-fold with minimal essential medium, and 0.1 ml thereof was inoculated into each well of the microculture plates. Three wells were used for a single sample. The plates were sealed and incubated at 35°C. Six days thereafter, the medium was washed off with phosphate-buffered saline; the cultures were irradiated with ultraviolet light for 10 min to kill the virus and then fixed and stained with 1% crystal violet solution in 20% ethanol. After thorough rinsing with water, the CPE was observed.

Plaque counting was performed as follows. One-tenth milliliter of the virus-compound mixture was put into each well of the culture plates, which were then sealed and kept at 35°C for 3 h. After removing the excess fluid, 0.1 ml of overlay medium, consisting of 1% methylcellulose and 2% heat-inactivated calf serum in Eagle minimal essential medium was added. The microplates were again sealed and incubated. Three or 4 days thereafter, the overlay medium was washed off with phosphate-buffered saline, and the cultures were ultraviolet irradiated, fixed, and strained. After rinsing with water, the plaques were counted under an ordinary light microscope at low magnification. The technical specifics were essentially the same as those reported previously from this laboratory (5).

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TABLE 1. List of disinfectants tested

Commonly used disinfectants	
1. Ethanol (99.5) ^a	
2. Isopropyl alcohol (95)	
3. Sodium hypochlorite (10)	
4. Formaldehyde (30)	
5. Phenol (99)	
6. Cresol (50)	
Surface-active detergents	
7. Benzalkonium chloride (10) (A, B, C, D, E) ^b	
8. Mixture of benzalkonium chloride (10) and polyethyleneglycol nonylphenyl ether (10)	
9. Mixture of cetyltrimethylammonium chloride (6.67) and benzalkonium chloride (3.33)	
10. Mixture of alkylpolyaminoethyl glycine (10) and polyethyleneglycol nonylphenyl ether (3)	
11. Alkylbenzyltrimethylammonium chloride (10)	
12. Sodium lauryldiaminoethyl glycine (10)	
13. Mixture of iodine (1.75) and polyethyleneglycol nonylphenyl ether (10)	
14. Tetradecyltrimethylammonium bromide (10)	
15. Polyoctylpolyaminoethyl glycine (6) and polyethyleneglycol nonylphenylether (4) (F, G) ^b	
16. Mixture of chlorohexidine digluconate (5) and polyethyleneglycol nonylphenyl ether (3.75)	
Chlorobenzene mixtures	
17. Mixture of <i>o</i> -dichlorobenzene (75) and cresol (10) (H, I) ^b	
18. Mixture of <i>o</i> -dichlorobenzene (57.96), cresol (16.98), and methanol (13.1)	
19. Mixture of <i>o</i> -dichlorobenzene (60), cresol (15), and methanol (5)	
20. Mixture of <i>o</i> -dichlorobenzene (70.7) and chlorocresol (7.3)	

^a Numbers in parentheses indicate percentage of active ingredient.

^b Products from different manufacturers.

RESULTS AND DISCUSSION

Preliminary experiments showed that the disinfectants, within the range of test concentrations, exerted little or no damage upon the culture cells. The viral CPE was clear 3 to 4 days after the inoculation of virus. Plaques, approximately 0.5 mm in diameter, were visible at the same time. Results obtained are summarized in Table 2, in which it is shown that compounds no. 3, 9, and 13 exhibit comparatively high effectiveness based on the minimum concentrations necessary to prevent CPE.

Using these compounds, together with some of the commonly used disinfectants, tests were carried out with the purified virus (see Table 3). Minimum concentrations required for the inactivation of purified virus were less than those used for the same virus in the crude state.

Virus inactivation kinetics curves were determined by the plaque counting method, and the representative data are illustrated in Fig. 1, which clearly reveals the minimum time and concentrations required for complete inactivation

TABLE 2. Effect of disinfectants upon infectivity of *variola virus*^a

Disinfectant no.	Concn (%)	No. of wells showing CPE/ no. of wells inoculated			
		1 min ^b	3 min	5 min	10 min
1	70-50	0/3	0/3	0/3	0/3
	40	3/3	0/3	0/3	0/3
	30	3/3	3/3	3/3	3/3
2	50-40	0/3	0/3	0/3	0/3
	30	3/3	3/3	0/3	0/3
	20	3/3	3/3	3/3	3/3
3	2.0-0.1	0/3	0/3	0/3	0/3
	0.05	3/3	3/3	1/3	1/3
4	1.0	0/3	0/3	0/3	0/3
	0.5	1/3	0/3	0/3	0/3
5	0.25	3/3	3/3	3/3	3/3
	2.0	0/3	0/3	0/3	0/3
6	1.0	3/3	3/3	3/3	3/3
	2.0-1.0	0/3	0/3	0/3	0/3
7 ^c	0.5	3/3	3/3	1/3	0/3
	1.0	0/3	0/3	0/3	0/3
8	0.5	3/3	3/3	0/3	0/3
	0.25-0.01	3/3	3/3	3/3	3/3
9	2.0	0/3	0/3	0/3	0/3
	1.0-0.5	3/3	3/3	3/3	3/3
10	2.0-0.25	0/3	0/3	0/3	0/3
	0.1	3/3	0/3	0/3	0/3
11	0.05	3/3	3/3	3/3	3/3
	2.0-1.0	0/3	0/3	0/3	0/3
12	0.5-0.25	3/3	3/3	3/3	3/3
	2.0-1.0	0/3	0/3	0/3	0/3
13	0.5-0.25	3/3	3/3	3/3	3/3
	2.0-0.1	0/3	0/3	0/3	0/3
14	0.05	3/3	3/3	3/3	3/3
	2.0-1.0	0/3	0/3	0/3	0/3
15 ^c	0.5	3/3	2/3	0/3	0/3
	0.25	3/3	3/3	3/3	3/3
16	2.0	0/3	0/3	0/3	0/3
	0.5-0.25	3/3	3/3	3/3	3/3
17 ^c	1.0-0.25	3/3	3/3	3/3	3/3
	2.0-1.0	0/3	0/3	0/3	0/3
18	0.5	3/3	3/3	3/3	2/3
	0.25	3/3	3/3	2/3	2/3
19	2.0-1.0	0/3	0/3	0/3	0/3
	0.5	3/3	2/3	0/3	1/3
20	0.25	3/3	3/3	3/3	3/3
	2.0	0/3	0/3	0/3	0/3
	1.0	2/3	0/3	0/3	0/3
	0.5	3/3	2/3	0/3	0/3
	0.25	3/3	3/3	3/3	3/3

^a Bandung strain virus: infective titer was 10⁷ plaque-forming units/ml on Jinet cell monolayer cultures under methylcellulose overlay medium.

^b Time of exposure.

^c Different samples from different manufacturers (see Table 1) were tested in parallel, and identical results were obtained.

tion of virus. Judged by the curves, compounds no. 3, 9, and 13 (all at 0.1%) were comparatively effective.

A series of experiments carried out in the

TABLE 3. Effect of selected disinfectants upon infectivity of purified variola virus^a

Disinfectant no.	Concn (%)	No. of wells showing CPE/no. of wells inoculated			
		1 min ^b	3 min	5 min	10 min
1	40	0/3	0/3	0/3	0/3
	30	2/3	0/3	0/3	0/3
	20	3/3	3/3	3/3	3/3
3	0.05	0/3	0/3	0/3	0/3
	0.025	3/3	2/3	0/3	0/3
	0.01	3/3	3/3	3/3	3/3
5	2.0	0/3	0/3	0/3	0/3
	1.0	3/3	3/3	3/3	3/3
9	0.1	0/3	0/3	0/3	0/3
	0.05	3/3	2/3	2/3	1/3
13	0.1	0/3	0/3	0/3	0/3
	0.05	2/3	0/3	0/3	0/3
	0.025	3/3	3/3	3/3	3/3

^a Bandung strain virus, purified by centrifugation; infective titer was 9.5×10^6 plaque-forming units/ml on Jinet cell monolayer cultures under methylcellulose overlay medium.

^b Time of exposure.

same way with the Harvey and Bandung strains of virus showed essentially the same results. Also there was no essential difference between the data obtained with variola virus and those obtained with vaccinia virus.

This paper describes experiments in which the effects of disinfectants on variola virus were examined by a microplate culture method, with CPE and plaque formation used as indicators of virus inactivation. The procedures adopted were straightforward, reproducible, and economically advantageous. Results obtained with two strains of virus of different origin were the same. Although no essential difference was noted in the disinfectant sensitivity of the variola and vaccinia viruses, the former are perhaps more suitable for smallpox disinfection studies than the latter. Among 20 kinds of compounds tested, several were found to be superior, as judged by the minimum effective concentrations. The values needed for purified virus were less than those needed for crude virus. The effects of disinfectants were more quantitatively analyzed by investigating the virus inactivation kinetics curves, determined by plaque counting. This technique seemed to be particularly useful for the purpose of this study and perhaps is the method of choice for virus disinfection research in general.

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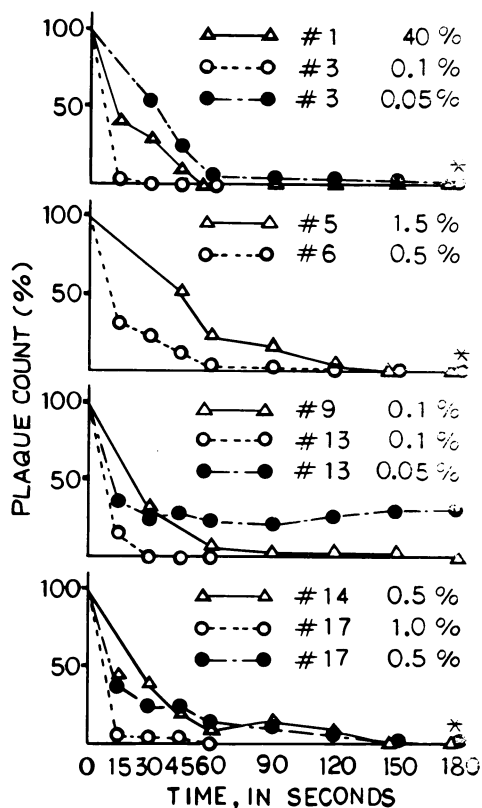


FIG. 1. Inactivation kinetics of variola virus by selected disinfectants (see Table 1) as determined by plaque counting. The ordinate indicates the average number of plaques from three inoculated wells, given as a percentage of those of the original inoculum; the abscissa indicates the time of exposure of virus to disinfectant, in seconds. Asterisk indicates the presence of residual active virus (range, one to three plaques) in all three wells).

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